Oil Palm Biotechnology
Recent achievements and prospects

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CIRAD
French Agricultural Research Centre for International Development
Montpellier, France

UNAL, Bogotà, Colombia, June 2008

Cirad in brief…

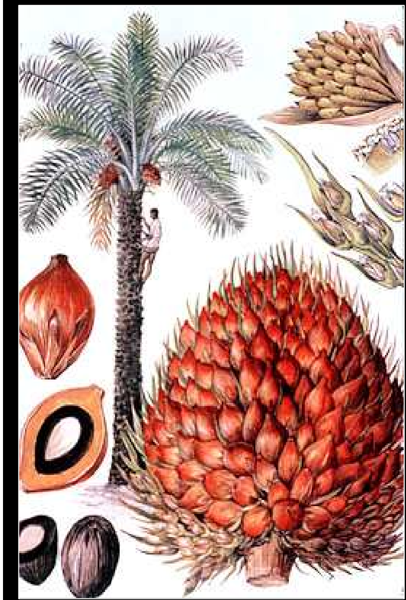
The Agricultural Research Centre for International Development, CIRAD, is a French agricultural research centre working for development in developing countries and the French overseas regions. Most of its research is managed under collaborative projects.

CIRAD has chosen sustainable development as the cornerstone of its operations worldwide. Research at Cirad takes account of the long-term ecological, economic and social consequences of changes in developing communities and countries.

CIRAD contributes to development through research and training, dissemination of information, innovation and appraisals. Its expertise spans life sciences, human sciences and engineering sciences and their application to agriculture and food, natural resource management and society.

CIRAD employs 1825 people, including 1047 senior staff members of whom 856 are scientists, and has an annual operating budget of 203 million euros.

The oil palm



- A giant perennial grass: Monocotyledoneous, *Arecaceae* (Palmaceae)

Coconut palm, date palm, rattan, edible palms,..

- Two cultivated species :
  - *Elaeis guineensis*
  - *Elaeis oleifera*

  (enriched in unsaturated FAs)
  - Interspecific hybrid

The oil palm

✓ 8 millions ha planted in intertropical regions

✓ The first world source of edible vegetable oil (ahead soya)

✓ A 8.3 billions USD business

✓ Imports in EU-15 : 1.6 billions USD

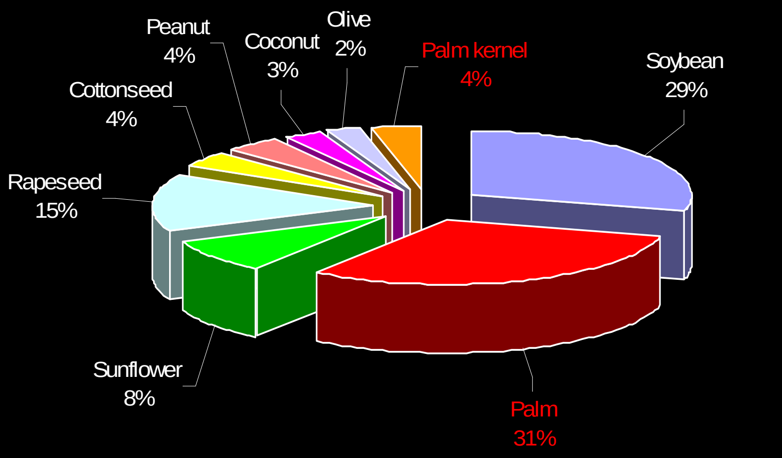
A strategic crop for tropical countries

Major Vegetable Oil: World Supply (Million Metric Tons)

	2002/2003	2003/2004	2004/2005	2005/2006	2006/2007
Soybean	30.57	29.97	32.28	34.11	34.94
Palm	27.71	29.59	33.88	35.37	37.37
Sunflowerseed	8.12	9.13	9.01	10.11	10.10
Rapeseed	12.21	14.14	15.73	17.07	17.61
Cottonseed	3.51	3.83	4.72	4.56	4.74
Peanut	4.62	5.01	5.06	5.18	5.00
Coconut	3.16	3.29	3.44	3.54	3.26
Olive	2.51	3.00	2.74	2.28	2.85
Palm Kernel	3.36	3.67	4.13	4.31	4.48

Source : USDA-FAS 08-2006

Major vegetable oils : world supply 2006/2007



Source : USDA-FAS 08-2006

Oil palm in Colombia

✓Colombia is the world's fifth producer of palm oil and the leading producer in Latin America

✓During the 1980's, the number of hectares of oil palm in Colombia has tripled.

✓By now, oil palm was the country's most important raw material in the production chain of oil seeds and oils and fats.

✓The areas with the highest number of hectares of oil palm are (in order): The departments of Meta (1), Cesar (2), Santander (3), Magdalena (4), Nariño (5), Casanare (6), Bolívar (7), Cundinamarca (8), Chocó (9) and Norte de Santander (10).

Source: Fedepalma

Facing the global context …

✓ The last 10 years have been marked by:

- a significant increase in demand for fat: + 50 %

- a twofold increase in the production of oil palm and palm kernel, which now account for one third of total vegetable fat production.

✓ This trend is likely to continue over the next few years:

In addition to traditional uses for vegetable fat, there is a increase interest and forecasted demand for bio-fuels.

Facing the global context …

✓ Meeting these demands will be extremely difficult, if not impossible, unless there is a considerable increase in oil palm production.

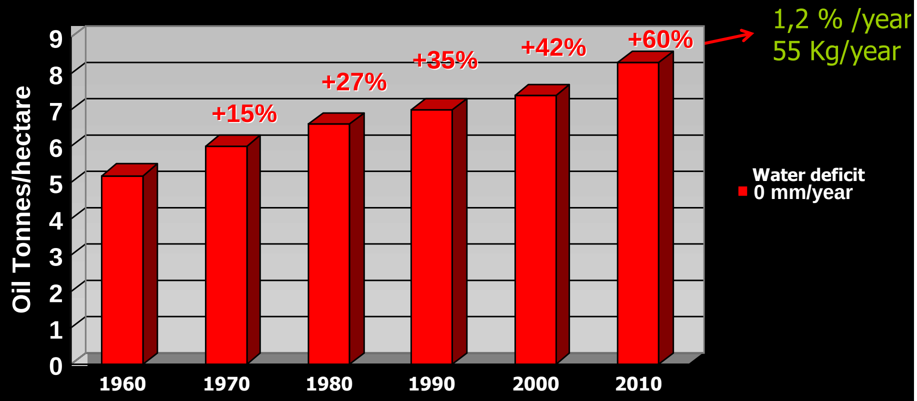
✓ The necessary increase in oil palm production will involve extending plantations but also improving yields.

✓ This will only be possible if planters can rely on quality plant material

In order to achieve this goal, it is important to establish production centres for high quality planting material, located in oil-producing regions

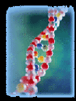
Overall genetic progress from 1960 to date

Genetic value of Cirad Seeds

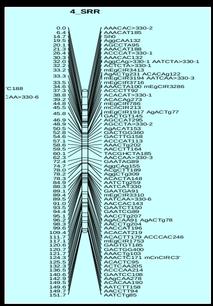


Biotechnologies are impacting breeding strategies

Molecular breeding



o Molecular analysis of genetic diversity in *E. guineensis* and *E. oleifera* germplasms
o Large scale development of PCR-based microsatellite markers
o Development of a reference high-density linkage map
o Genome mapping and QTL (Quantitative Trait Loci) detection :



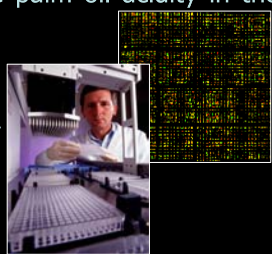
* Resistance to *Fusarium* wilt
* Increased and stable oil palm production
* Detection and introgression of *E. oleifera* genetic factors conferring resistance to Bud Rot.

A network of field trials is being established in order to validate QTL markers, to implement marker-assisted breeding strategies and to pursue physical mapping towards the characterisation, cloning and tagging of useful genes.

Biotechnologies are impacting breeding strategies

Structural and Functional genomics:

Aims
Cloning genes of agronomic interest such as the *Sh* major gene responsible of the fruit variety or
Cloning a gene coding for a lipase responsible of the palm oil acidity in the pulp of mature fruits.



Tools
(i) EST (Expressed Sequence Tag) of cDNA sequences,
(ii) Physical mapping of BAC clones,
(iii) cDNA-AFLP mapping
(iv) Differential display of cDNAs

The aim is to assemble an extensive catalogue of oil palm genes, which can be screened either on the basis of their sequence affinities (similarity to known genes of interest) or by using high throughput macro- or microarray screening to monitor their expression patterns.

Oil palm micropropagation

- Feasibility of SE-based process
  - 2 millions vitroplants
  - Sizeable genetic progress
- Transferred in producing countries: Indonesia, Malaysia, Côte d'Ivoire, Costa Rica, Colombia
- Bottlenecks from scaling-up:
  - Production costs: 2 to 4 US$ per vp (5 to 7 x seeds)
  - Genetic fidelity

Oil production in clonal palms

(with reference to standard cross L2T x D10D)

	*3-5 years period**			*Adult period***		
	FFB	extraction rate	oil	FFB	extraction rate	oil
Average	105 %	108 %	114 %	98 %	108 %	105 %
Selection 1/5	124 %	112 %	136 %	114 %	112 %	128 %

Bottlenecks to commercial development

1. Production costs

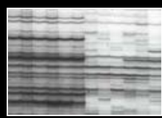
To set up an improved production process for oil palm

- ❖ large scale production (x 10^5 vitroplants / year / clonal line)
- ❖ significant reduction in manpower costs

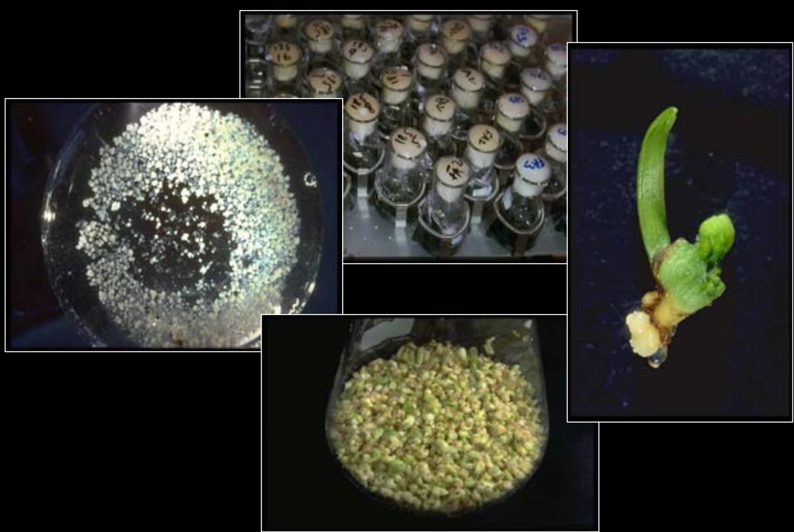
2. Clonal fidelity

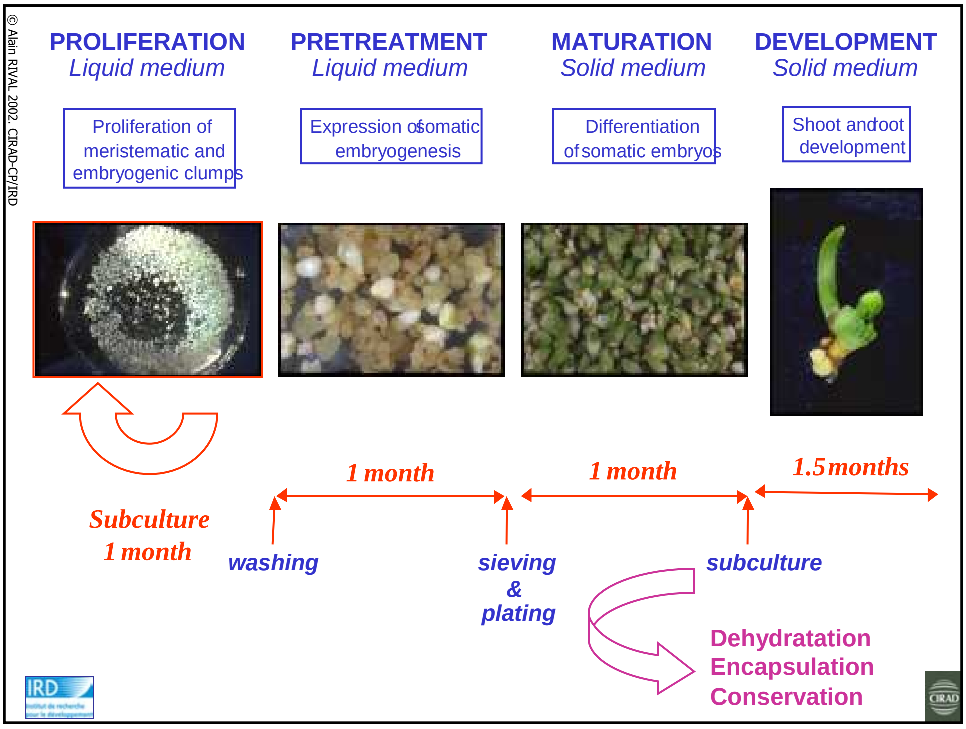
To set up a set of DNA/RNA/serological markers

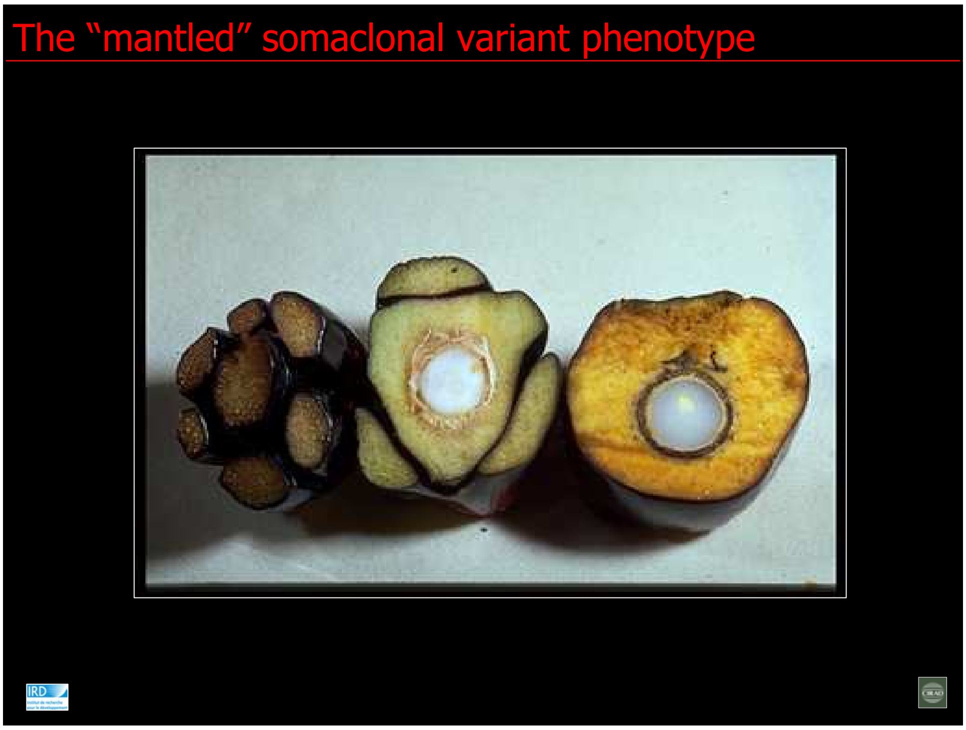
- ❖ monitoring of SE process
- ❖ discard off-type lines as early as possible



Oil palm embryogenic suspensions




© Alain RIVAL 2002. CIRAD-CP/IRD
PROLIFERATION
Liquid medium
PRETREATMENT
Liquid medium
MATURATION
Solid medium
DEVELOPMENT
Solid medium
Proliferation of meristematic and embryogenic clumps
Expression of somatic embryogenesis
Differentiation of somatic embryos
Shoot and root development
1 month
1 month
1.5 months
Subculture
1 month
washing
sieving
&
plating
subculture
Dehydratation
Encapsulation
Conservation
IRD
CIRAD


The "mantled" somaclonal variant phenotype
IRD
CIRAD

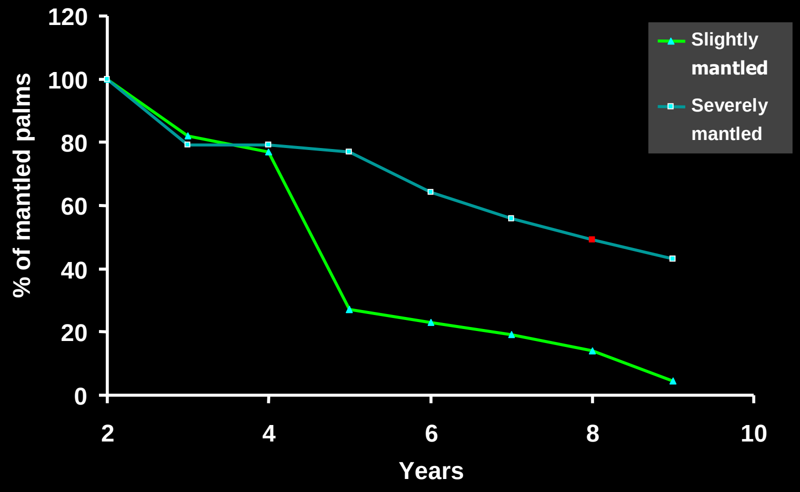
Characteristics of the "mantled" somaclonal variation

- ✓ Inter clonal variability: 0 to 85%
- ✓ Intra clonal variability: between production batches
- ✓ Variable expression on a given palm :
  from : one fruit on one single bunch
  to : all the fruits from all the bunches
- ✓ Expression varying with time
  - 100% of the slightly mantled palms reverted to the normal phenotype after 10 years in the field
  - 50% of severely mantled reverted to normal
- ✓ Non-Mendelian sexual transmission

Impact of the "mantled" somaclonal variation

	Observed palms	Normal palms	Slightly abnormal	Severely abnormal
IDEFOR Côte d'Ivoire	29,415	90.3%	3.7 %	6.0 %
FELDA Malaysia	18,935	92.0%	5.6 %	2.4 %
IOPRI Indonesia	6,771	87.3%	5.3 %	7.4 %

Reversion of somaclonal variation in the field



Data from CNRA La Mé Research Station (Côte d'Ivoire)

A few things that we know … (from the field)

Recent in-depth analysis of phenotypic characters suggested that "normal" regenerants may show reversible developmental abnormalities:

- Flower sex-ratio
- Flower abortions
- Vegetative growth

The impact of somaclonal variation in oil palm clones is wider than the "mantled" phenotype alone …

A few things that we know … (from the field)

✓ The "mantled phenotype occurs very rarely in progenies originating from sexual reproduction (a handful of individuals in 500 millions commercial seeds sold yearly…)

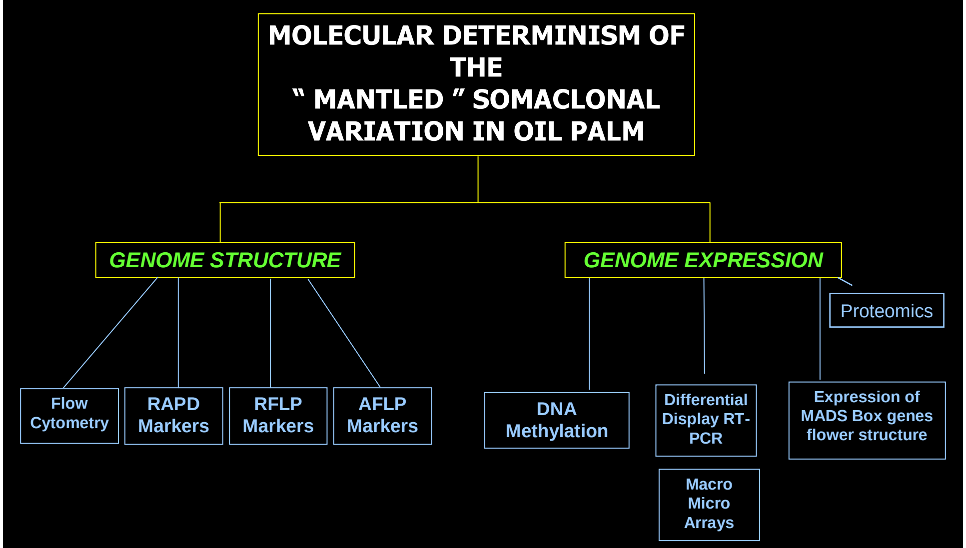
✓ One spontaneous ecotype of *Elaeis guineensis* showing a stable "mantled" phenotype has been described and named "*poissonii*"

✓ Several different SE protocols gave rise to the same variant phenotype

✓ Recloning from leaf explant sampled on variant somaclones always gives rise to variant somaclones

✓ Somaclonal variants in Date Palm (*Phoenix dactylifera*) originating from SE are reported to show supernumerary carpels

Research strategy at palm Develpment Group - UMR1097



A few things that we know … (from the tissue culture lab)

Oil Palm embryogenic calli

Nodular Compact Callus

Fast Growing Callus

In vitro regeneration *via* Somatic Embryogenesis

SOMACLONAL VARIANTS < 5%

SOMACLONAL VARIANTS 100%

What is (are) the underlying molecular mechanism (s) ?

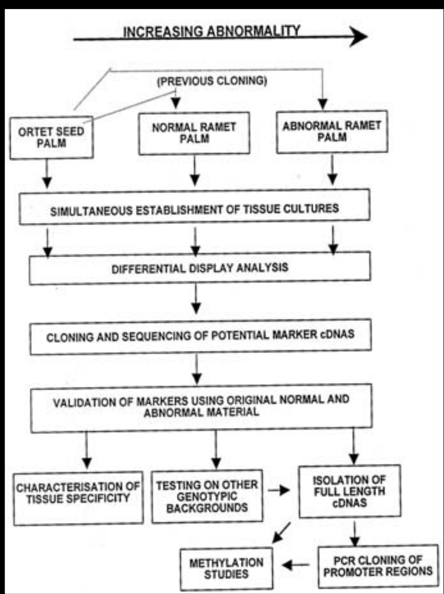
- ✓ Genome size
- ✓ DNA markers
  - RAPDs
  - AFLPs
- ✓ Gene expression markers
  - - ddRT-PCR
  - - Homeotic MADS Box RFs
- ✓ DNA Methylation studies
  - ✓ Global methylation rates
  - ✓ Methylation-sensitive RFLP/AFLP
  - ✓ DNA methyltransferases
  - ✓ Chromatin remodelling

Flow cytometric analysis

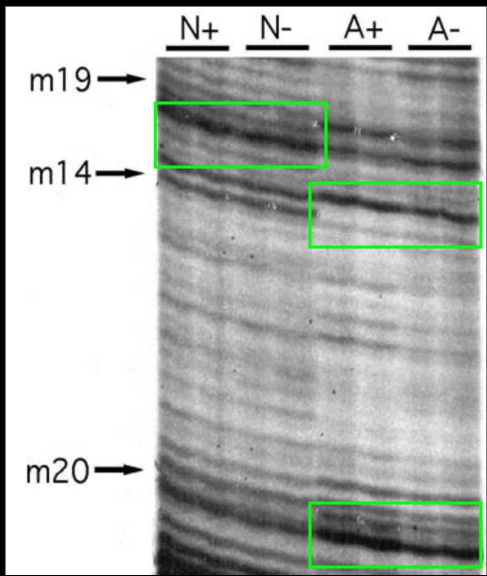
Plant material	qDNA (pg/nucleus)
Seed derived palms	3.786 ± 0.125^{b}
Acclimatized vitroplants	3.701 ± 0.223^{b}
In vitro rooted plantlets	3.790 ± 0.164^{b}
Fast Growing Calli	3.295 ± 0.379^{a}
Nodular Compact Calli	3.290 ± 0.432^{a}
Friable Calli	3.212 ± 0.223^{a}

Plant Cell Reports. 16: 884-887

Strategy for the identification of expression markers



Differential Display analysis of shoot apices



N = ortet-derived material
A = abnormal ramet-derived material
- = plantlets grown on hormone-free medium
+ = plantlets grown on medium containing 10^{-5}M BAP

Journal of Experimental Botany, 53 : 1387-1396.

Results of the ddRT approach

- 5 expression markers potentially exploitable in clonal conformity testing (4 A-specific, 1 N-specific)
- Potential markers for all developmental stages (callus, somatic embryo, leafy shoot (apex), plantlet leaf
- Quantitative difference between N & A varies
- Identification of possible factors of importance, including wound response (*EGAD1*)

Tree Physiology : 26, 585–594.

A collaborative project funded by the Malaysian Oil Palm Board

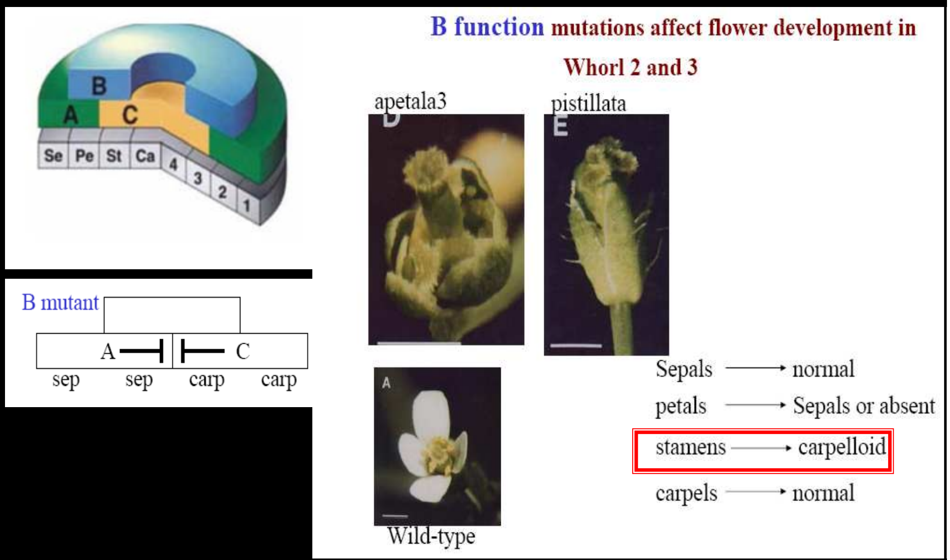
Search for markers through MacroArrays

- ✓ 2000 cDNAs /filter
- ✓ cDNAs originating from SSH libraries (variant/normal in vitro cultivated material)
- ✓ Tissues from key stages of regeneration through embryogenic suspensions
- ✓ 48 putative markers in embryogenic suspensions
- ✓ 12 putative markers in SE-derived shootlets

Egad1 Patent. MPOB/CIRAD

Northern blot analysis of a "mantled" specific probe

Flower MADS Box homeotic genes



Flower MADS Box homeotic genes

- ✓ The "mantled" abnormality involves a homeotic modification of the floral organs (cf apetala3)
- ✓ Genes potentially regulating flower development are being isolated using PCR and cDNA library screening with heterologous probes
  - Identification of 15 MADS Box genes from 7 different groups (SQUA, DEF, GLO, AG et AGL2) in oil palm
  - Changes in expression related to the expression of the « mantled » phenotype

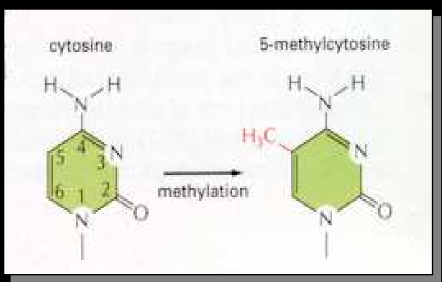
J Mol Evol (2006) 62:15–31

The DNA methylation hypothesis

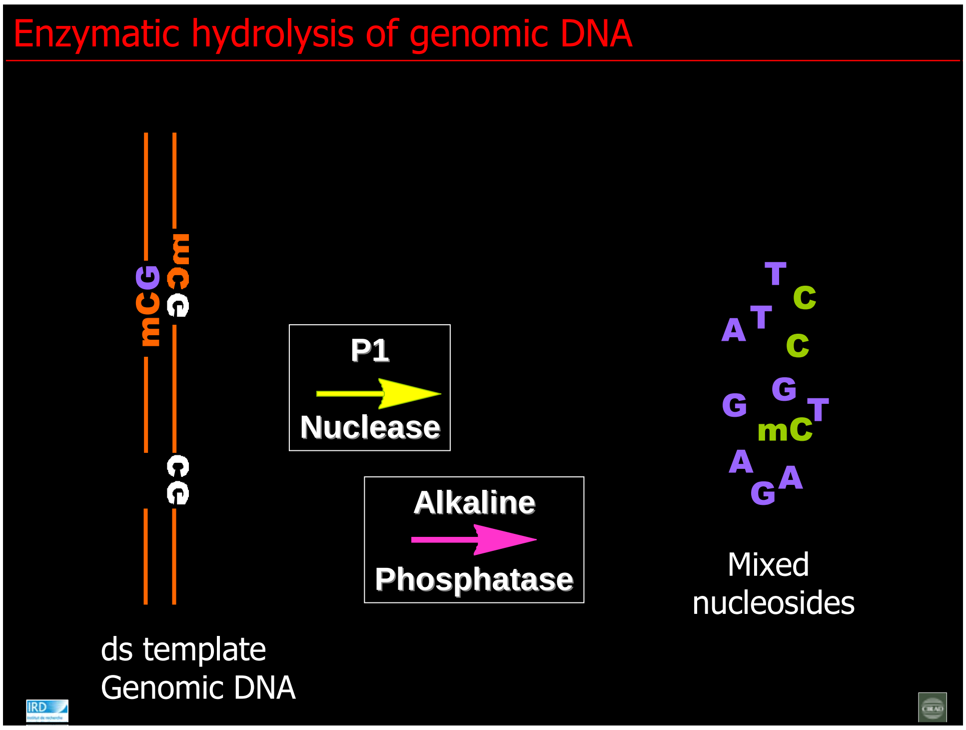
- ✓ Epigenetic nature of the *mantled* abnormality
    - Field results
    - Standard DNA markers (RAPDs, AFLPs ….)
- ✓ DNA methylation rates changes with developmental stages
- ✓ Tissue culture induced instability
- ✓ Growth regulators (2,4-D) affect DNA methylation rates
- ✓ Defects in DNA methylation (anti MET1) generated abnormal flower phenotypes in *Arabidopsis*

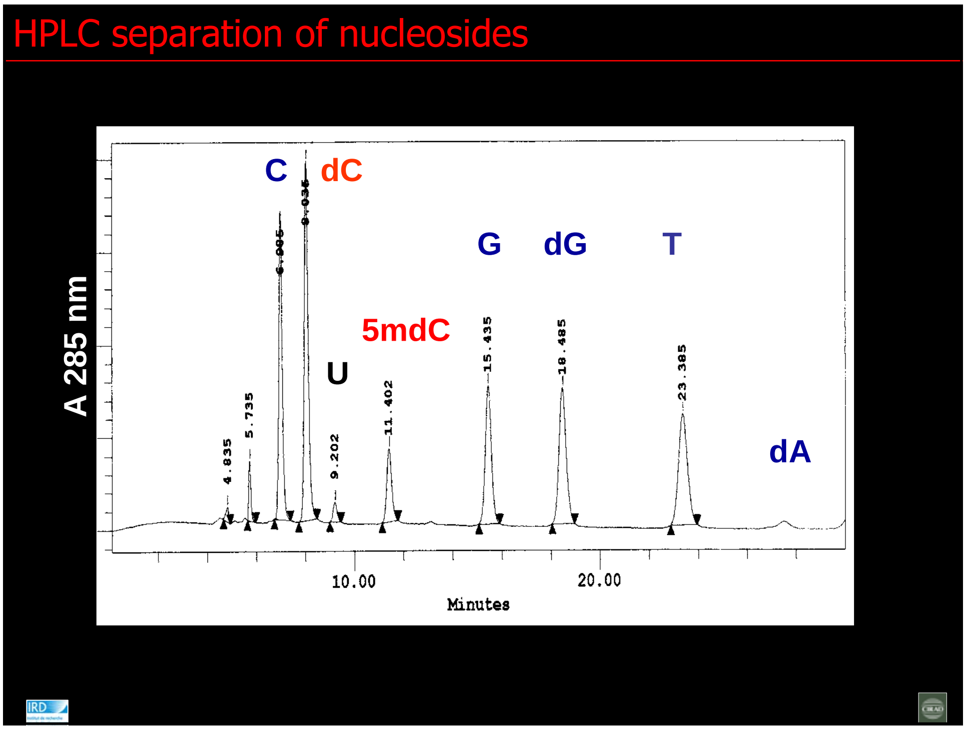
Plant Breeding. 117(1), 73-76.

Estimation of Global DNA Methylation Rates

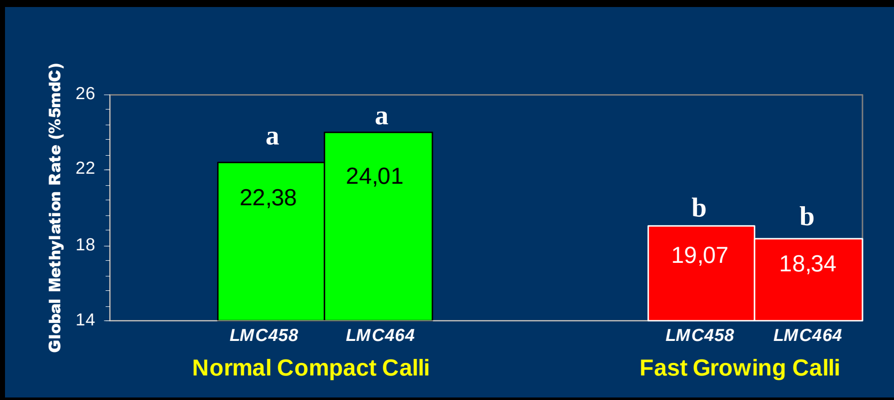


$$\frac{5mdC}{dC + 5mdC}$$


Enzymatic hydrolysis of genomic DNA
mCG
CG
P1
Nuclease
Alkaline
Phosphatase
T
C
A
T
C
G
G
T
mC
A
G
A
Mixed
nucleosides
ds template
Genomic DNA


HPLC separation of nucleosides
A 285 nm
C
dC
G
dG
T
5mdC
U
dA
4.835
5.735
9.202
11.402
15.435
18.485
23.385
10.00
20.00
Minutes

Global Methylation Rates in embryogenic calli

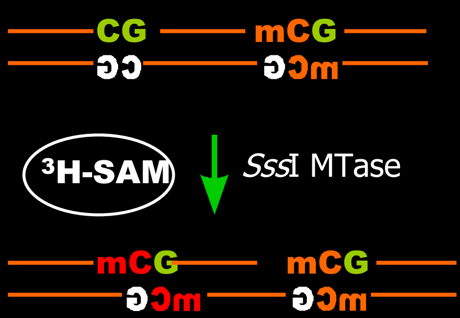


No Clone effect; Type effect : F(1,11) = 58.19; p<0.0000

Plant Cell Rep. 19 (7): 684-690.

SssI-Methylase Accepting Assay

Saturation of CG methylatable sites



$$\text{Radioactivity } \alpha = \frac{1}{\%\text{CpG Meth}}$$

E. Oakeley & J.P Jost

*Sss*I-Methylase Accepting Assay

Normalised Methylation Index (NMI) calculated from *Sss*I-MAA analysis of DNA extracted from embryogenic calli

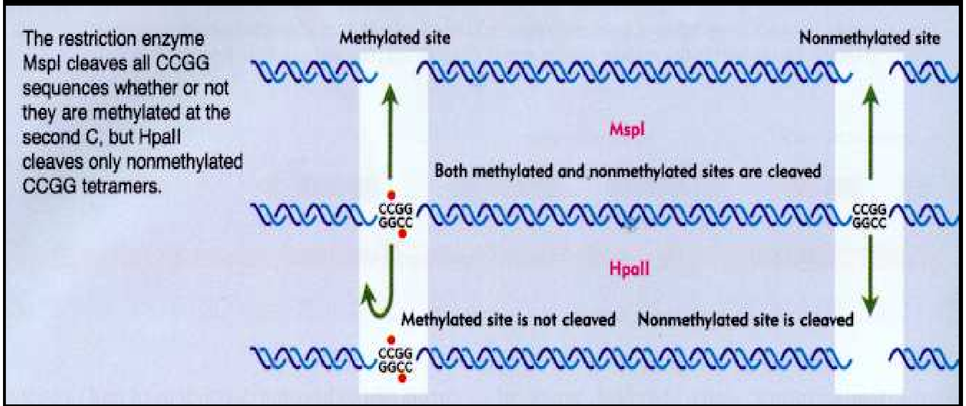
Clonal line	Callus type	NMI ± SD
LMC458	NCC	0.60 ± 0.16^{a}
	FGC	0.99 ± 0.04^{b}
LMC464	NCC	0.73 ± 0.09^{a}
	FGC	1.17 ± 0.43^{b}

Each NMI is the mean of three independent measurements.
Data followed by the same letter are not significantly different at the 5% level.

Search for MS-RFLP Markers

- Oil palm cDNA probes from immature inflorescences and/or calli
- Isoschizomeric restriction enzymes (*Msp*I/*Hpa*II)
- Search for differential genomic DNA Methylation patterns

Isoschizomeric restriction enzymes

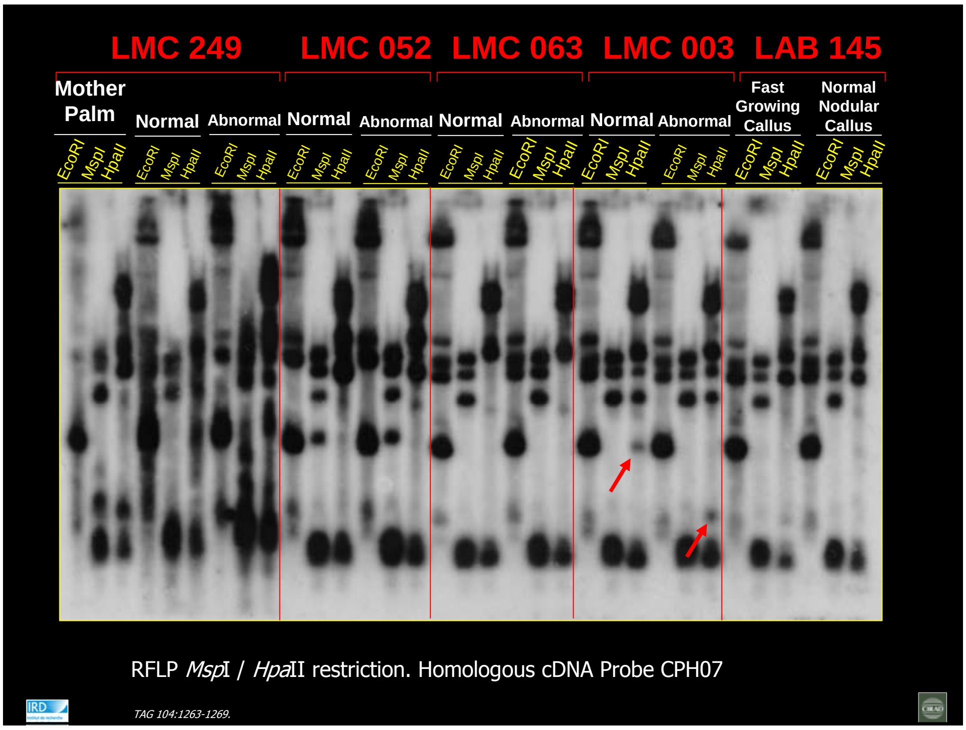


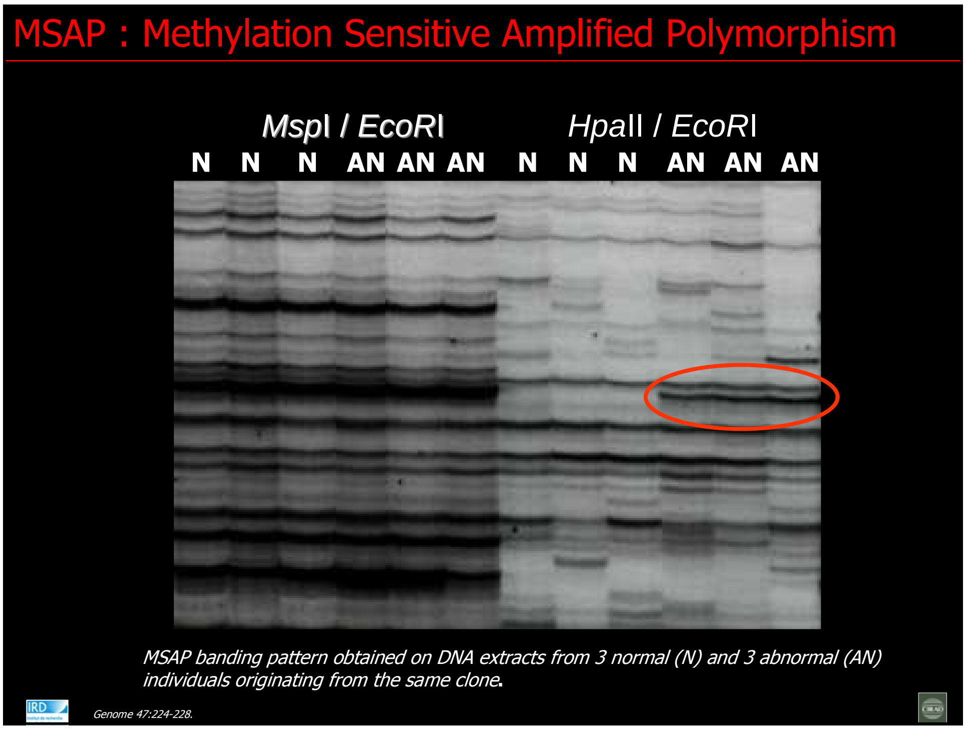
Search for MS-RFLP Markers

LMC 458 | LMC 464

MspI *HpaII* *MspI* *HpaII* *MspI* *HpaII* *MspI* *HpaII*

Southern blot experiments involving RFLP digestion products hybridised with the cDNA probe CPH062.


LMC 249
LMC 052
LMC 063
LMC 003
LAB 145
Mother Palm
Normal
Abnormal
Normal
Abnormal
Normal
Abnormal
Normal
Abnormal
Fast Growing Callus
Normal Nodular Callus
EcoRI MspI HpaII
RFLP *Msp*I / *Hpa*II restriction. Homologous cDNA Probe CPH07
TAG 104:1263-1269.


MSAP : Methylation Sensitive Amplified Polymorphism
MspI / EcoRI
HpaII / EcoRI
N N N AN AN AN N N N AN AN AN
MSAP banding pattern obtained on DNA extracts from 3 normal (N) and 3 abnormal (AN) individuals originating from the same clone.
Genome 47:224-228.

VARIOMETH
EXPLORING THE ROLE OF DNA METHYLATION IN EPIGENETIC VARIATION IN HIGHER PLANTS

- The VARIOMETH fellowship will focus on the role of DNA methyltransferases on the determinism of somaclonal variation and on the exploration of the relationship between DNA methylation and chromatin remodelling.
- Both approaches will be developed in parallel with the aim of describing specific molecular events which could be used for the development of markers of epigenetic instability in plants.
- These markers will be integrated in a strategy aimed at the identification of *in vitro* treatments which are prone to generate epigenetic variability in somatic embryogenesis-based micropropagation processes.

European Commission
Human Resources and Mobility
Marie Curie Outgoing International Fellowship
2004-2007

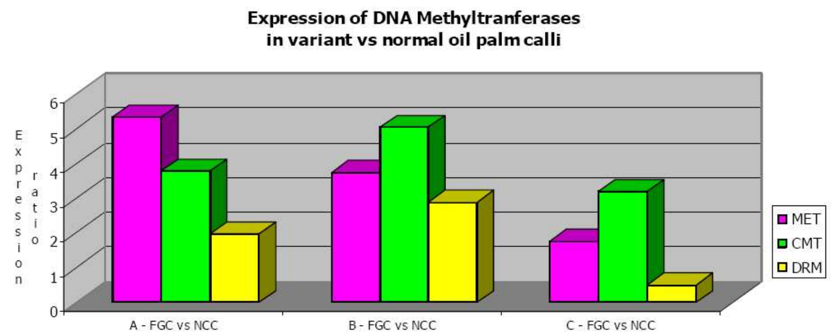
VARIOMETH:
EXPLORING THE ROLE OF DNA METHYLATION IN EPIGENETIC VARIATION IN HIGHER PLANTS

Model plant	Sequences	MET	CMT	DRM
Oryza sativa	% Identity	65	62	55
	% Positives	79	75	70
	Accession #	AAP44671.1	AAN60988.1	ABF93591.1
Zea mays	% Identity	67	64	54
	% Positives	81	76	68
	Accession #	AAC16389.1	AAK11516.1	AAF68437.1
Arabidopsis thaliana	% Identity	57	55	53
	% Positives	71	70	69
	Accession #	AAA32829.1	AAK69756.1	AAF66129.1
Nicotiana tabaccum	% Identity	62	59	59
	% Positives	76	73	74
	Accession #	BAF36443.1	BAC53936.1	BAC67060.1

Homologies of oil palm DNA Methyltransferases with available accessions from model plants

J. Exp. Bot (2008) in press

VARIOMETH:
EXPLORING THE ROLE OF DNA METHYLATION IN EPIGENETIC VARIATION IN HIGHER PLANTS



Quantitative Real Time PCR was performed on total RNAs extracted from embryogenic calli from three different genotypes (A, B and C). For each genotype, Fast-Growing Calli (FGC, generating 100% of "mantled" palms) were compared to Nodular Compact Calli (which yield on average 5% of variant palms). Gene expression was represented by the ratio of expression in FGC by expression in NCC.
For each methyltransferases gene, expression levels were standardized using control Elongation Factor EgEF1-α1 sequence from oil palm (accession no. AY550990) as a reference.

J. Exp. Bot (2008) in press

VARIOMETH:
EXPLORING THE ROLE OF DNA METHYLATION IN EPIGENETIC VARIATION IN HIGHER PLANTS

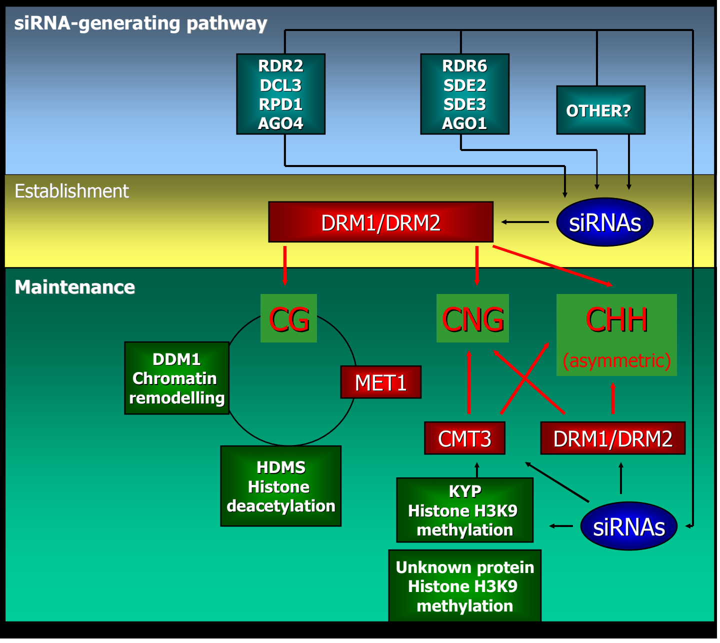
Conclusions

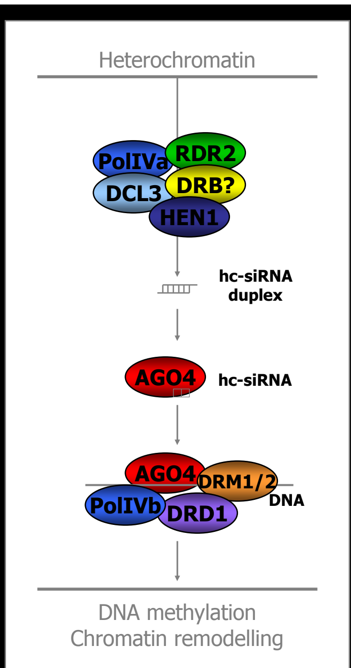
1. Full lengths cDNAs coding for three different DNA (cytosine-5)-methyltransferases families (namely MET, CMT and DRM) were isolated from oil palm (*Elaeis guineensis* L) and the corresponding EgMET, EgCMT and EgDRM products were studied.

2. Global DNA hypomethylation which was previously measured in variant calluses is not related with any decrease in expression of any of the three isolated METases

Establishment and Maintenance of DNA methylation

GARDENING THE GENOME
DNA METHYLATION IN
ARABIDOPSIS THALIANA
Simon W.-L. Chan*, Ian R. Henderson* and Steven E. Jacobsen





✓ Methyltransferases are known to be involved in sRNA-mediated gene regulation

✓ Methyltransferases are part of active DNA-protein complexes

✓ The abundance of transcripts is not sufficient to prove the activity of Methyltransferases

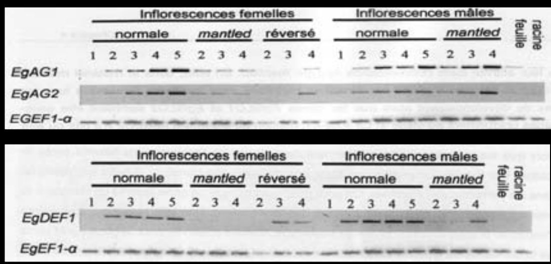
✓ Post-transcriptional / translational regulation are involved

Post-transcriptional small RNA pathways in plants: mechanisms and regulations
Hervé Vaucheret
Genes & Dev. 2006 20: 759-771
doi:10.1101/gad.1410506

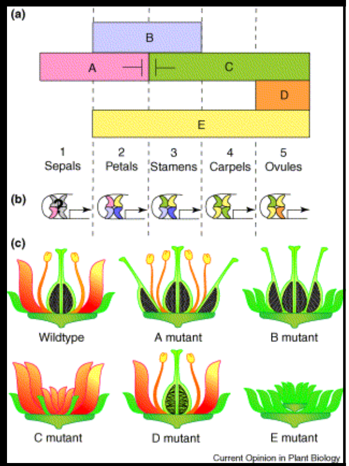
Research work under way

✓ The *ABC* model involves genes governing flower structure in higher plants, known as MADS box genes.

✓ Oil palm MADS box genes have been found to be affected in *mantled* material



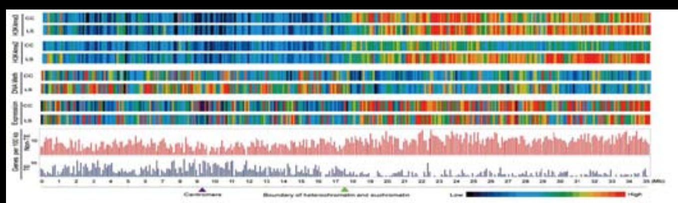
Reduced expression of genes Eg DEF1(B type), EgAG2 (C and D type) in abnormal oil palm flowers



Present research work is focusing on the study of DNA methylation in and around MADS box genes (promoter/intron/coding sequences)

Future research work

✓ Recent advances have facilitated the mapping of DNA methylation across the entire genome in model plants (*Arabidopsis*, rice).



✓ Tools are now in hand to determine whether tissue specific or developmental patterns of gene expression are dictated by changes in DNA methylation.

✓ Deep sequencing technology will be applied for the discrimination of differentially methylated sequences in mantled *vs* normal material

Our final aim is to isolate candidate sequences which can be used for the development of Methylation Sensitive detection kits for the early identification of variant cell lines

Relevant literature

JALIGOT E., RIVAL A., BEULÉ T., DUSSERT S. & VERDEIL J.-L. (2000) Somaclonal variation in Oil Palm (*Elaeis guineensis* Jacq.): The DNA methylation hypothesis. *Plant Cell Reports 19 (7): 684-690.*

TREGEAR J., MORCILLO F., RICHAUD F., BERGER A., SINGH R., CHEAH S.C., HARTMANN C., RIVAL A. & DUVAL Y. (2001) Characterisation of a defensin gene expressed in oil palm inflorescence: induction during tissue culture and possible association with epigenetic somaclonal variation events*. Journal of Experimental Botany*, *53 : 1387-1396.*

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JALIGOT E., BEULÉ T., BAURENS F.C., BILLOTE N. & RIVAL A. (2004). MSAP screening for differentially methylated markers associated with the « mantled » somaclonal variation in oil palm (*Elaeis guineensis* Jacq.). *Genome 47:224-228.*

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ADAM H,JOUANNIC S, ESCOUTE J., DUVAL Y , VERDEIL J-L & J.W. TREGEAR (2005) Reproductive developmental complexity in the african oil palm (Elaeis guineensis, Arecaceae). *American Journal of Botany* 92(11): 1836–1852.

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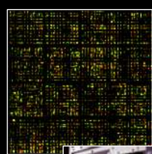
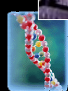
Acknowledgements



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